

Isolation and Genetic Characterization of H5N2 Influenza Viruses from Pigs in Korea[▽]

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Received 20 November 2008/Accepted 3 February 2009

Due to dual susceptibility to both human and avian influenza A viruses, pigs are believed to be effective intermediate hosts for the spread and production of new viruses with pandemic potential. In early 2008, two swine H5N2 viruses were isolated from our routine swine surveillance in Korea. The sequencing and phylogenetic analysis of surface proteins revealed that the Sw/Korea/C12/08 and Sw/Korea/C13/08 viruses were derived from avian influenza viruses of the Eurasian lineage. However, although the Sw/Korea/C12/08 isolate is an entirely avian-like virus, the Sw/Korea/C13/08 isolate is an avian-swine-like reassortant with the PB2, PA, NP, and M genes coming from a 2006 Korean swine H3N1-like virus. The molecular characterization of the two viruses indicated an absence of significant mutations that could be associated with virulence or binding affinity. However, animal experiments showed that the reassortant Sw/Korea/C13/08 virus was more adapted and was more readily transmitted than the purely avian-like virus in a swine experimental model but not in ferrets. Furthermore, seroprevalence in swine sera from 2006 to 2008 suggested that avian H5 viruses have been infecting swine since 2006. Although there are no known potential clinical implications of the avian-swine reassortant virus for pathogenicity in pigs or other species, including humans, at present, the efficient transmissibility of the swine-adapted H5N2 virus could facilitate virus spread and could be a potential model for pandemic, highly pathogenic avian influenza (e.g., H5N1 and H7N7) virus outbreaks or a pandemic strain itself.

The infection of swine with influenza A viruses is of significant importance to the swine industry and to the epidemiology of human influenza. The severity of the clinical symptoms appears to be dependent upon the infecting virus strain, the age and immune status of the animal, and the presence of concomitant pathogens or environmental stress factors. Swine influenza viruses (SIVs) also cause respiratory diseases in humans, and several instances of the zoonotic transmission of SIV from pigs have been reported (13, 22, 36, 43). The ability of the influenza virus to cross between animal species is controlled by the viral genes, and the prevalence of transmission depends on the species involved.

Domesticated pig species are known to allow the productive replication of both avian and human influenza viruses. This susceptibility is due to the presence of both α -2,3- and 2,6-galactose-sialic acid (sialyloligosaccharide) linkages of cellular receptors lining the pig trachea (22). Successful transmission between animal species can follow genetic reassortment in which a progeny virus containing a specific gene constellation has the ability to replicate in the new host. Under experimental

conditions, pigs are susceptible to infection with a range of avian and human influenza viruses (29), and though not often documented, the interspecies transmission of avian viruses to pigs in nature has been reported. Pigs in Europe and China have been infected with avian H1N1 viruses (14, 37), and a purely avian H4N6 influenza virus caused a disease outbreak in pigs in Canada (27). In China, Peiris et al. reported on swine infections with avian H9N2 influenza virus, and since then, frequent exposure to this subtype has been documented (34, 41). Lately, reassortant viruses between avian H5N1 and H9N2 subtypes caused pig diseases and death in some parts of China (6, 7, 57), and in the United States, reassortant H2N3 viruses (between American avian-like and contemporary swine triple reassortant-like) recently have been identified (32).

In Korea, four subtypes (H1N1, H1N2, H3N1, and H3N2) of SIVs have been reported in the pig population (23–25, 49). Phylogenetic analyses have indicated that the Korean isolates were closely related to the triple-reassortant SIVs recently isolated from pigs in the United States containing internal segments derived from human (PB1), swine (NP, M, and NS), and avian (PB2 and PA) sources (23, 24, 47, 50). More recently, Pascua et al. reported that under vaccine pressure, a new serogroup of H3N2 SIVs had genetically evolved among Korean swine (39).

We now describe the isolation and characterization of two novel H5N2 swine influenza viruses, A/Swine/Korea/C12/08

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[▽] Published ahead of print on 18 February 2009.

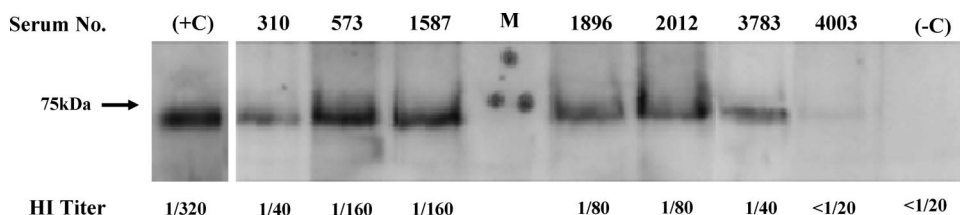


FIG. 1. Immunoblotting using H5 antigen derived from the Sw/Korea/C13/07 (H5N2) virus as the primary antibody and purified by ultracentrifugation with a CaCl_2 cushion at $112,600 \times g$ for 3 h at 4°C (Beckman). Thirty micrograms of H5 protein per lane was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and immunoblotted with pig sera at a dilution of 1:200. All of the sera giving a positive result in the HI and virus-neutralizing antibody tests were subjected to immunoblotting together with a random selection of sera giving negative HI results. A representative result indicating the different HI titers obtained for each respective serum is shown. M, 75-kDa protein marker (Bio-Rad); +C, positive control; -C, negative control.

(Sw/Kor/C12/08) and A/Swine/Korea/C13/08 (Sw/Kor/C13/08), which were isolated from pigs in South Korea. Genetic characterization showed that these viruses have high homology to avian H5N2 influenza viruses recently circulating among wild birds in Korea (our unpublished data) with the exception of the NP, M, PA, and PB2 genes of the Sw/Kor/C13/08 isolate, which have high homologies to those of the H3N1-like virus that circulated in pigs in Korea in 2006. To investigate the pathogenic potential of the novel H5N2 porcine viruses in mammalian hosts, we conducted animal challenge experiments in pig and ferret models. Furthermore, seroprevalence in swine sera from 2006 to 2008 suggested that the avian H5N2-like H5 viruses have been infecting swine since 2006, even though there was no large-scale outbreak in swine herds in South Korea.

MATERIALS AND METHODS

Viruses. The viruses used in this study were obtained from 7- to 8-week-old pigs showing typical clinical symptoms of influenza-like illness from the Chungnam province of South Korea. The two swine influenza viruses, A/Swine/Korea/C12/08 (Sw/Kor/C12/08) and A/Swine/Korea/C13/08 (Sw/Kor/C13/08), were isolated from nasal swabs or lung specimens by the inoculation of supernatants and tissue homogenates into monolayers of Madin-Darby canine kidney (MDCK) cells. The subtypes of Sw/Kor/C12/08 and Sw/Kor/C13/08 were determined by two multiplex reverse transcription-PCR (RT-PCR) assays and confirmed by sequencing as previously described (3).

Sequencing and phylogenetic analysis. Viral RNA was extracted from cell culture isolates using a QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA). RT-PCR was performed under standard conditions using influenza-specific primers (4, 19). The nucleotide sequencing of the amplified products was done using a DNA sequencer (Model 377; Applied Biosystems, Perkin-Elmer, Foster City, CA) and a *Taq* Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequences were resolved using the ABI PRISM collection program (Perkin-Elmer, Foster City, CA). The DNA sequences were compiled and edited using the Lasergene sequence analysis software package (DNASTAR, Madison, WI). Multiple sequence alignments were made using Clustal_X (1, 53). The rooted phylograms were prepared using the neighbor-joining (NJ) algorithm and then plotted using the program NJ plot (42). The trees presented in Fig. 2a to h are based on the full-length nucleotide sequences of each gene segment.

Pig serologic tests. A hemagglutination inhibition (HI) assay was performed to determine the seroprevalence of the novel H5N2 viruses from 2006 to May 2008. A total of 4,108 sera collected from major swine production provinces were tested for the presence of H5 antibody. All sera were heat inactivated at 56°C for 30 min and pretreated with receptor-destroying enzyme (RDE) from *Vibrio cholerae* (Denka Seiken, Tokyo, Japan) to remove nonspecific serum inhibitors. The sera then were tested for H5 antibody by the HI technique with 0.5% chicken red blood cells (38). A neutralization test was done on selected HI-positive sera to confirm results, as described previously (18). Neutralizing antibody titers were expressed as the reciprocal of the highest dilution of the sample that completely inhibited hemagglutination. Hemagglutination assays were per-

formed according to WHO/World Organization for Animal Health recommendations.

Western blotting. Western immunoblotting was done using H5 viral antigen derived from the formalin-inactivated H5N2 (Sw/Korea/C13/07) isolate that had been purified by ultracentrifugation with a CaCl_2 cushion at $112,600 \times g$ for 3 h at 4°C (5). Thirty micrograms of H5 protein per lane were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and immunoblotted with pig sera at a dilution of 1:200. All pig sera that gave positive reactions in the H5N2 HI and virus-neutralizing antibody tests were tested together with a random selection of at least five sera giving negative reactions (Fig. 1).

Replication and transmission of swine isolate. The experimental infection of pigs and ferrets with the H5N2 viruses was done in biosafety level 2 (BSL 2) and 3 (BSL 3+) containment facilities, respectively. Yorkshire white weanling pigs (approximately 5 to 6 weeks old) that were found to be free of detectable influenza virus by serologic testing were inoculated intranasally with 3.3×10^6 50% egg infective dose (EID_{50}) of virus in a volume of 1.0 ml (divided between two plastic syringes for the separate inoculation of each nostril). The swine H5N2 viruses were inoculated into two groups, and three uninoculated littermates were housed in an isolator with two inoculated pigs to test for the pig-to-pig transmission of virus. The pigs' temperatures and food consumption were recorded daily beginning 2 days before inoculation and ending 14 days after inoculation (the end of study). Each nostril was swabbed 2, 5, 7, and 9 days after inoculation, and virus subsequently was titrated in embryonated chicken eggs. The same procedure was done in 17-week-old male ferrets to study the transmissibility of the viruses in this host. Two experimental groups (each comprised two inoculated and two naïve-contact littermate ferrets) were inoculated intranasally with 3.3×10^6 EID_{50} of virus in 0.5 ml sterile phosphate-buffered saline under isoflurane anesthesia. To monitor virus shedding, nasal washes were collected from all ferrets every other day for 14 days and were titrated in embryonated chicken eggs.

Histopathology. Trachea, serum, lung, liver, intestine, spleen, and kidney from pigs and ferrets were collected on day 5 after inoculation. To prevent cross-contamination, different sterile instruments were used for collecting each tissue (5). Collected tissues were fixed in 10% neutral-buffered formalin. Immunohistochemistry was performed to examine the distribution of SIV antigens in the lung and other organs in infected animals using a goat polyclonal antibody against type A influenza virus nucleoprotein (Abcam, Cambridge, MA).

Nucleotide sequence accession numbers. The GenBank accession numbers assigned to the sequences determined in this study are FJ461592 to FJ461607.

RESULTS

Analysis of clinical samples. Nasal swabs and lung samples were submitted to the Chungbuk National University between 2004 and 2008 for the diagnosis of SIV infections. Average swine farms contain approximately 200 sows and 2,100 fattening pigs, according to the Korean Swine Association, and most have adopted farrow-to-finish operations (26). The Sw/Kor/C12/08 swine influenza virus was isolated from nasal swabs obtained from a 6-week-old Yorkshire white weanling pig from the Chungnam province through routine virus surveillance in

TABLE 1. Sequence homology of each gene from the two porcine H5N2 viruses with reference virus sequences available in GenBank^a

Gene	Sw/Kor/C12/08				Sw/Kor/C13/08			
	% Identity	Virus with the highest degree of sequence identity (accession no.)	Subtype	Phylogenetic lineage	% Identity	Virus with the highest degree of sequence identity (accession no.)	Subtype	Phylogenetic lineage
HA	99.7	Ga/SanJiang/160/06 (EF634332)	H5N2	Avian	99.8	Ga/SanJiang/160/06 (EF634334)	H5N2	Avian
NA	99.8	Ga/SanJiang/160/06 (EF634332)	H5N2	Avian	99.5	Ga/SanJiang/160/06 (EF634334)	H5N2	Avian
PB2	96.7	Dk/Denmark/65047/04 (DQ251450)	H5N2	Avian	99.5	Sw/Kor/CN22/06 (DQ923521)	H3N1	Swine
PB1	97.6	Dk/Denmark/65047/04 (DQ251450)	H5N2	Avian	97.8	Dk/Denmark/65047/04 (DQ251450)	H5N2	Avian
PA	98.4	Ck/Taiwan/165/99 (DQ376806)	H6N1	Avian	99.7	Sw/Kor/CN22/06 (DQ923517)	H3N1	Swine
NP	98.8	Dk/Hokkaido/Vac-3/07 (AB355930)	H5N1	Avian	99.8	Sw/Kor/CN22/06 (DQ923513)	H3N1	Swine
M	98.5	Dk/Jiang Xi/6568/04 (EF597301)	H4N6	Avian	99.7	Sw/Kor/CN22/06 (DQ923511)	H3N1	Swine
NS	99.8	Dk/Hokkaido/120/01 (AB286880)	H6N2	Avian	99.4	Dk/Hokkaido/120/01 (AB286880)	H6N2	Avian

^a The numbers in parentheses are GenBank accession numbers for the reference virus sequences. Ck, chicken; Dk, duck; Ga, garganey; and Sw, swine.

January 2008. In the same province in February 2008, the Sw/Kor/C13/08 swine influenza virus was isolated from lung tissues obtained from an 8-week-old Yorkshire white weanling pig with typical influenza-like symptoms, such as high fever, nasal discharge, coughing, and decreased food consumption. The subtypes of Sw/Kor/C12/08 and Sw/Kor/C13/08 were found to belong to the H5N2 family by two multiplex RT-PCR assays and sequencing as previously described (3). No other subtypes of swine influenza viruses were detected from the specimens submitted on the same days.

Sequence and phylogenetic analysis. Except for the PB2, PA, NP, and M genes of Sw/Kor/C13/08, sequence analysis revealed that each of the RNA segments of the two porcine H5N2 viruses have high nucleotide homology to avian viruses of the Eurasian lineage (Table 1). The nucleotide analysis of the PCR products indicated that Sw/Kor/C12/08 and Sw/Kor/C13/08 shared >99.2% nucleotide similarities in the HA, NA, PB1, and NS genes. This high genomic identity in surface genes and in some internal genes suggests that the same avian virus is the progenitor of the two isolates. Interestingly though, gene segments 1 (PB2), 3 (PA), 5 (NP), and 7 (M) of the Sw/Kor/C13/08 isolate have high homology with Sw/Kor/CN22/06-like (H3N1) swine influenza viruses, indicating that reassortment has occurred.

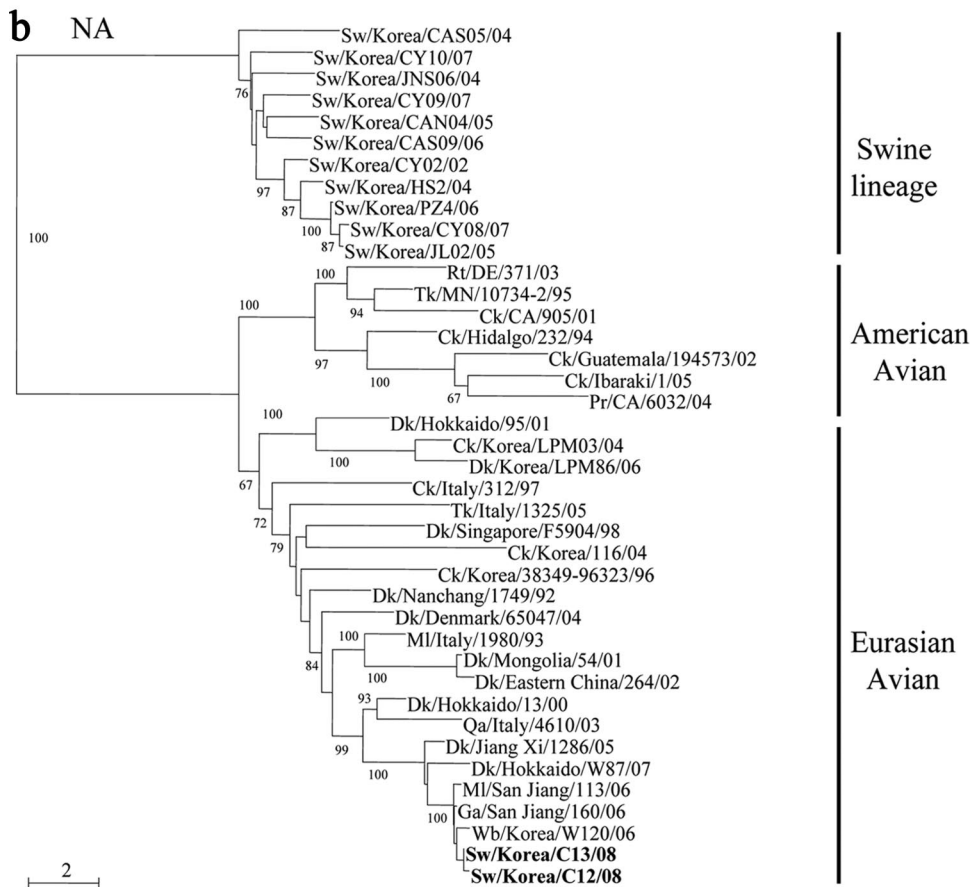
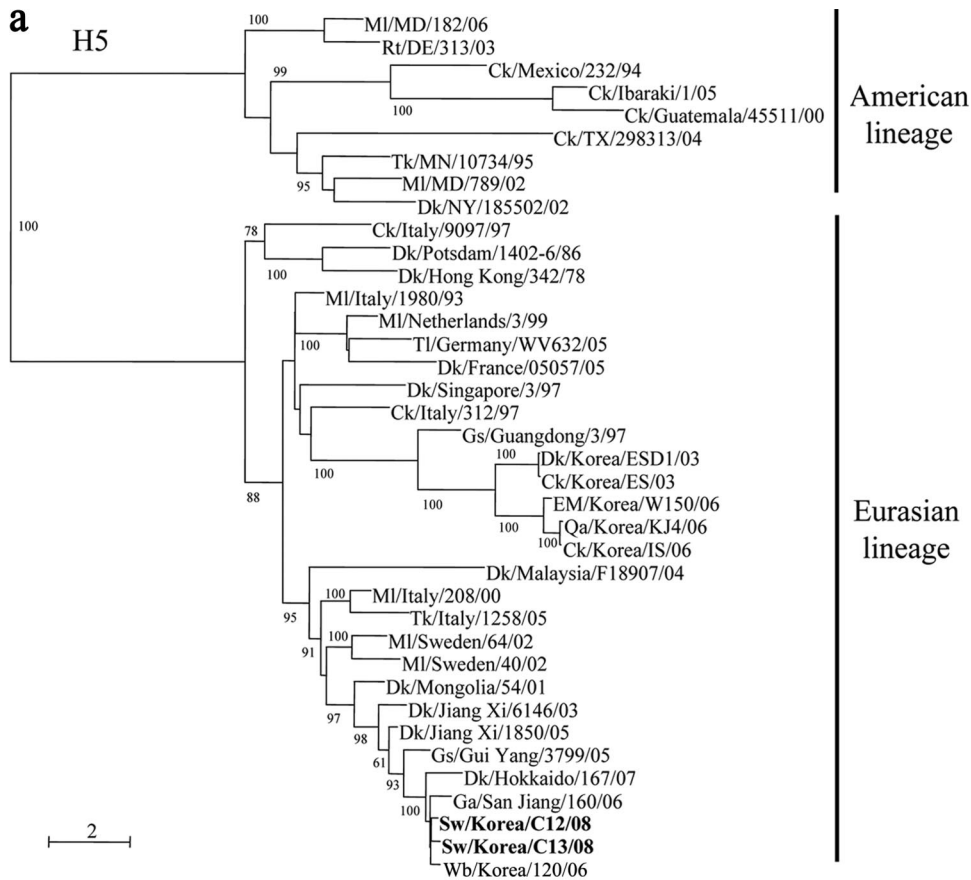
To understand the evolutionary relationship and origin of the Sw/Kor/C12/08 and the Sw/Kor/C13/08 viruses in detail, the phylogenetic alignment of the full-length gene sequences was performed (Fig. 2a to h). Phylogenetic analysis showed that the HA genes of both swine H5N2 isolates clustered together and had high nucleotide identities with low-pathogenicity avian influenza (LPAI) H5 viruses from China, specifically Ga/San Jiang/160/06 (>99%), rather than to recent highly pathogenic avian influenza H5N1 viruses present among domestic poultry (represented by Ck/Kor/IS/06; 88.6%) and migratory birds (represented by Em/Kor/W150/06; 89.3%) from Korea. The phylogeny of the NA genes paralleled what was observed for their HA gene comparisons, in which the two swine isolates still belonged to the same avian Ga/San Jiang/160/06-like lineage (>99% sequence homologies) (Fig. 2a and b). Apparently, Korean avian H5N2 influenza viruses isolated from wild birds in 2006 were also descendants of the Ga/San Jiang/160/06-like lineage (our unpublished data), indicating a common avian H5N2 virus precursor.

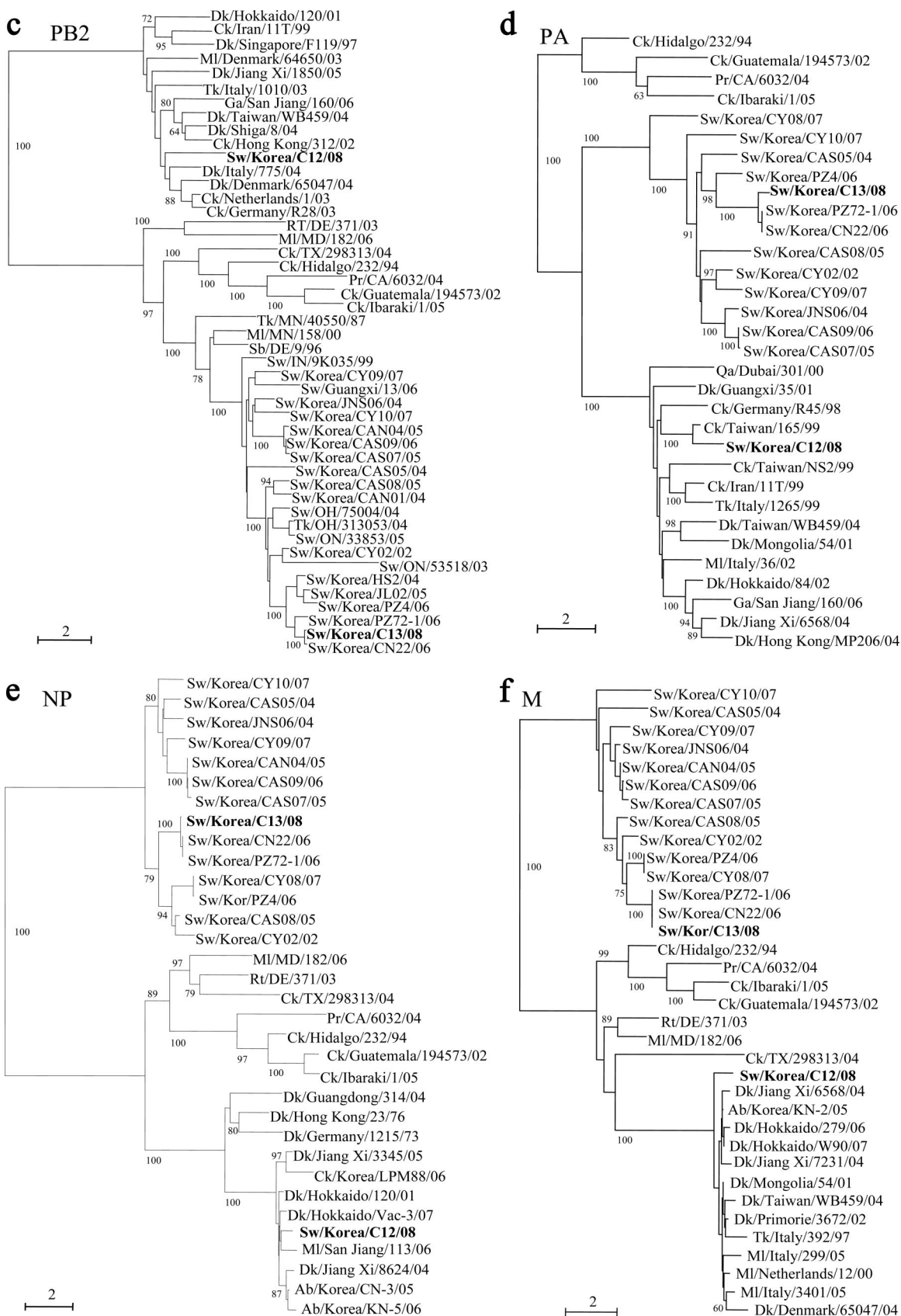
Phylogenetic analysis of the internal genes clearly showed the differences between the two porcine influenza virus iso-

lates, supporting the sequence analysis. The phylogenetic analysis revealed that each of the internal genes of Sw/Kor/C12/08 had a close relationship with different subtypes of LPAI isolates that are entirely of Eurasian phylogenetic lineage, indicating that these segments were derived from multiple virus sources. For the internal genes of the Sw/Kor/C13/08 virus, only the PB1 and NS genes were derived from avian origin, clustering together with the Sw/Kor/C12/08 virus (Fig. 2g and h). Phylograms of segments 1, 3, 5, and 7 of the virus diverged from avian to swine lineages and strongly indicated that they were derived from Sw/Kor/CN22/06-like (H3N1) swine influenza viruses, which were reportedly circulating in Korean pigs in 2006 (Fig. 2a to f) (47). These results show that the Sw/Kor/C12/08 isolate is a wholly avian virus, whereas Sw/Kor/C13/08 is an avian-swine reassortant from a possible common precursor. The analysis of Sw/Kor/C13/08 suggested that some of the avian H5N2 viruses that infected Korean pigs had undergone genetic reassortment with a swine influenza virus, probably due to the coinfection of the two influenza virus strains in the same pig.

Antigenic analysis and Western blotting. To investigate the prevalence of swine infection with the H5N2 viruses, swine sera collected from 2006 to May 2008 from at least four swine production provinces in Korea were tested. Of the 4,108 pig serum samples surveyed, 35 (0.85%) were positive for the swine H5N2 viruses by the HI assay (Table 2). As early as January 2006, positive serum samples already were obtained, with titers ranging from 40 to 160 HI units. The prevalence rate was only about 1.04% in 2006 but was down to around 0.74% in 2007 and 0.77% in 2008. Collectively though, the serologic data indicated that viral exposure was substantially higher during the winter months.

To further confirm the specificity of the HI test results, virus neutralization tests were conducted. To test for the presence of neutralizing antibodies against H5 viruses, selected HI-positive pig sera were diluted twofold and mixed with 100 50% tissue culture infective doses of Sw/Kor/C13/08 or Sw/Kor/C12/08. In addition, an H5N1 virus (EM/Kor/W149/06) also was used to test the specificity of the antibodies present. Results showed that neutralizing antibodies against the swine H5N2 viruses correlated with the titers obtained from the HI assays (Table 2). Similar neutralization titers were obtained using a wild bird H5N2 virus. In contrast, lower neutralizing antibody titers (a difference of about two- to fourfold) were observed in the





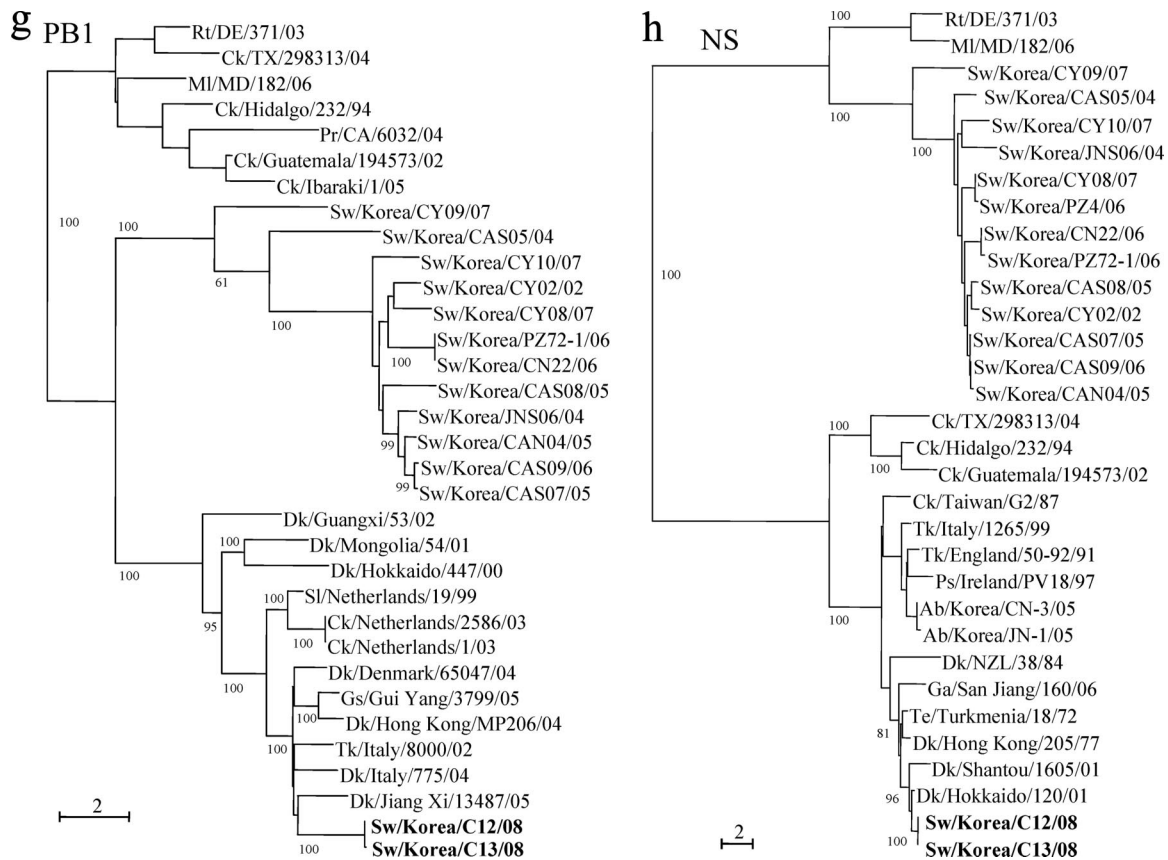


FIG. 2. (a and b) Phylogenetic tree based on the nucleotide sequences of the H5 HA gene (a) and the N2 NA gene (b). The nucleotide sequences were aligned using Clustal_X (1, 53), and the phylograms were generated by the NJ method using the tree-drawing program NJ plot (42). The scale represents the number of substitutions per nucleotide. Branch labels record the stability of the branches during 100 bootstrap replicates. Only bootstrap values $\geq 60\%$ are shown in each tree. The isolates in boldface type are the Korean swine H5N2 viruses being characterized in this study. Ck, chicken; Dk, duck; Ga, garganey; Gs, goose; MI, mallard; Qa, quail; RT, rudder turnstone; Tk, turkey; Tl, teal; and Wb, wild bird. Standard abbreviations are used for state names in the United States. (c to h) Phylogenetic tree based on the nucleotide sequences of the PB2, PA, NP, M, PB1, and NS genes. The nucleotide sequences were aligned using Clustal_X (1, 53), and the phylograms were generated by the NJ method using the tree-drawing program NJ plot (42). The scale represents the number of substitutions per nucleotide. Branch labels record the stability of the branches during 100 bootstrap replicates. Only bootstrap values $\geq 60\%$ are shown in each tree. The isolates in boldface type are the Korean swine H5N2 viruses being characterized in this study. Ab, aquatic bird; Pr, parrot; Ps, pheasant; Sb, shorebird; SI, shoveler; Sw, swine; and Te, tern. Standard abbreviations are used for state names in the United States.

swine sera when an avian H5N1 virus was used (data not shown).

H5 antigen against the Sw/Kor/C13/08 virus, which was purified by ultracentrifugation at $112,600 \times g$ for 3 h at 4°C , was used in the Western blot analysis. The immunoblotting results correlated with the results of the HI and virus-neutralizing tests (Fig. 1 and Table 2). These findings showed that anti-H5 antibodies were present in several pigs and that the H5N2-like viruses could naturally infect pigs, although the incidence of such infection is low, as shown by the seroprevalence survey (Table 2) and the absence of a reported large-scale outbreak in swine herds due to this subtype.

Animal experiments. Two pigs were infected with H5N2/08 viruses, and three contact pigs were added 1 day later in the same isolators and housed together until the end of the experiments. Mild cough, nasal discharge, and elevated body temperature were observed on days 2 through 7 in all inoculated pigs, but no acute respiratory signs were observed, such as lethargy and dyspnea (data not shown). All of the viruses were

recovered from nasal swabs obtained from the inoculated pigs after intranasal infection. On day 2 after inoculation, the mean virus titer of nasal swabs was $\geq 2.3 \log_{10} \text{EID}_{50}/0.1 \text{ ml}$. Virus was shed for at least 5 days (Table 3). Virus titers in nasal swabs peaked on day 5 at 2.5 to $3.33 \log_{10} \text{EID}_{50}/0.1 \text{ ml}$. The infection of the wholly avian-derived virus (Sw/Kor/C12/08) persisted for as long as 5 days but was not transmitted to any of the three contact pigs. However, two of the three littermates of the avian-swine reassortant (Sw/Kor/C13/08) were positive for virus transmission on day 5, with mean titers of 2.7 and $2.3 \log_{10} \text{EID}_{50}/0.1 \text{ ml}$ (Table 3). The pigs inoculated with Sw/Kor/C13/08 showed higher nasal swab titers, persisting for as long as 7 days, than those inoculated with the wholly avian-derived isolate (Table 3).

With the variable replication kinetics and transmissibility in pigs of the two H5N2 porcine isolates, we sought to determine whether such results could also be obtained in mammalian hosts by using two groups of ferrets (two inoculated and two contact animals) as experimental models. All 17-week-old fer-

TABLE 2. Serologic reactivity to H5N2 influenza viruses for pig sera collected from Korean swine farms^a

Yr and mo	No. of sera tested	No. (%) of sera positive for H5 antibody in HI tests	HI/NT titer(s) of positive sera
2006			
January	155	4 (2.58)	40/20, 160/80, 160/80, 80/80
February	156	3 (1.92)	80/40, 80/80, 40/40
March	151	1 (0.64)	40/20
April	135	2 (1.48)	160/80, 80/80
May	139	1 (0.71)	80/40
June	129	1 (0.77)	40/20
September	118	0	
October	143	0	
November	149	1 (0.67)	40/40
December	163	2 (1.22)	40/20, 80/40
2007			
January	181	3 (1.65)	80/40, 160/80, 40/40
February	213	2 (0.93)	40/20, 40/40
March	221	5 (2.26)	40/20, 40/20, 80/40, 80/80, 40/20
April	208	0	
May	216	2 (0.92)	40/40, 40/20
June	201	0	
September	186	0	
October	193	0	
November	192	1 (0.52)	40/40
December	209	2 (0.95)	40/20, 160/160
2008			
January	218	2 (0.91)	40/40, 40/80
February	223	2 (0.89)	160/80, 40/80
March	209	1 (0.47)	80/40
Total	4,108	35 (0.85)	

^a The sera giving positive HI test results were confirmed by Western blotting using H5 antigen (Fig. 1) and neutralization (NT) tests (Table 2).

rets used for the study were found to be seronegative at day 0 for antibodies against swine influenza H1N1, H1N2, H3N1, and H3N2 viruses and avian H5N2 viruses by the HI assay. For the Sw/Kor/C12/08 virus, nasal washes were positive for virus (2.7 log₁₀ EID₅₀/0.1 ml) infection only at 2 days after inoculation, whereas a higher viral titer was observed 2 days after inoculation (3.4 log₁₀ EID₅₀/0.1 ml) in Sw/Korea/C13/08-infected ferrets and persisted until day 7 (Table 3). Thus, the virus titer obtained from an Sw/Kor/C12/08-infected mammalian host possibly were traces of the infection load and were not

due to successful virus replication, but the Sw/Korea/C13/08 virus did actively replicate in infected ferrets. No observable clinical signs of influenza-like disease were noted in the infected ferrets, and neither the wholly avian (Sw/Kor/C12/08) nor the avian-swine reassortant (Sw/Kor/C13/08) virus was transmitted among the contact ferrets. These results, therefore, indicate that the Sw/Kor/C13/08 isolate bearing some swine gene segments (M, NP, PA, and PB2) can readily infect both animal models and is efficiently transmissible via contact in swine hosts, as opposed to the purely avian Sw/Kor/C12/08 isolate. However, neither H5N2 virus could be transmitted in ferrets.

Histopathologic examination. To investigate the tissue distribution of viruses, we collected samples of trachea, serum, lung, liver, intestine, spleen, and kidney from an infected pig and ferret 5 days after inoculation from each group. The lungs were positive for virus in all infected pigs and those of contact pigs of the Sw/Kor/C13/08 virus-infected group (Fig. 3). Therefore, the H5N2 viruses can persist in pigs for at least 5 days after inoculation. However, the lungs were positive for virus only in Sw/Kor/C13/08-infected ferrets (data not shown). No virus was recovered from the liver, intestine, spleen, or kidney of any pig or ferret. Lung tissue collected on day 5 showed that Sw/Kor/C12/08 caused less severe lung and tracheal tissue damage than Sw/Kor/C13/08 (Fig. 3).

Molecular analysis. The deduced amino acid sequences of the viral proteins were investigated to examine the molecular characteristics of the viruses in detail and to identify possible determinants of interspecies transmission from birds to pigs. The alignment of the amino acid sequences of the surface proteins revealed that the two Korean swine H5N2 isolates have >98.2 and >98.9% sequence identity to the reference avian isolate (Ga/San Jiang/160/06) in HA and NA while sharing about 98.7 and 99.1% homology with each other in these respective viral proteins. At the HA1-HA2 cleavage site, both of the porcine isolates had a Q-R-E-T-R motif in the connecting peptides that is typical for LPAI H5 viruses (9), confirming their low-pathogenicity pathotypes. All of the isolates had a glutamine at position 222 (position 226 in H3 numbering) and a glycine residue at position 224 (position 228 in H3 numbering), indicating a preferential binding of sialic acids to the sugar chain by an α 2-3 linkage (Table 4). The potential glycosylation sites in the HA1 domain and proposed receptor binding sites were conserved in both isolates. None of the porcine

TABLE 3. Nasal excretion of H5N2 viruses in pigs and ferrets

Day	Swab viral titer (log ₁₀ EID ₅₀ /0.1 ml) from ^a :							
	Swine				Ferret			
	Sw/Kor/C12/08		Sw/Kor/C13/08		Sw/Kor/C12/08		Sw/Kor/C13/08	
	Inoculated	Contact	Inoculated	Contact	Inoculated	Contact	Inoculated	Contact
-1	—	—	—	—	—	—	—	—
2	2.5	—	2.7	—	2.7	—	3.4	—
5	2.5	—	3.3	2.7	—	—	2.7	—
7	—	—	1.3	1.3	—	—	0.7	—
9	—	—	—	—	—	—	—	—

^a All viruses and animal experiments were handled in BSL 2 (swine infections) or BSL 3+ (ferret inoculation) containment facilities approved by the Korea Centers for Disease Control and Prevention, and the research staff wore fitted HEPA filter masks. The dash marks indicate no detectable virus.

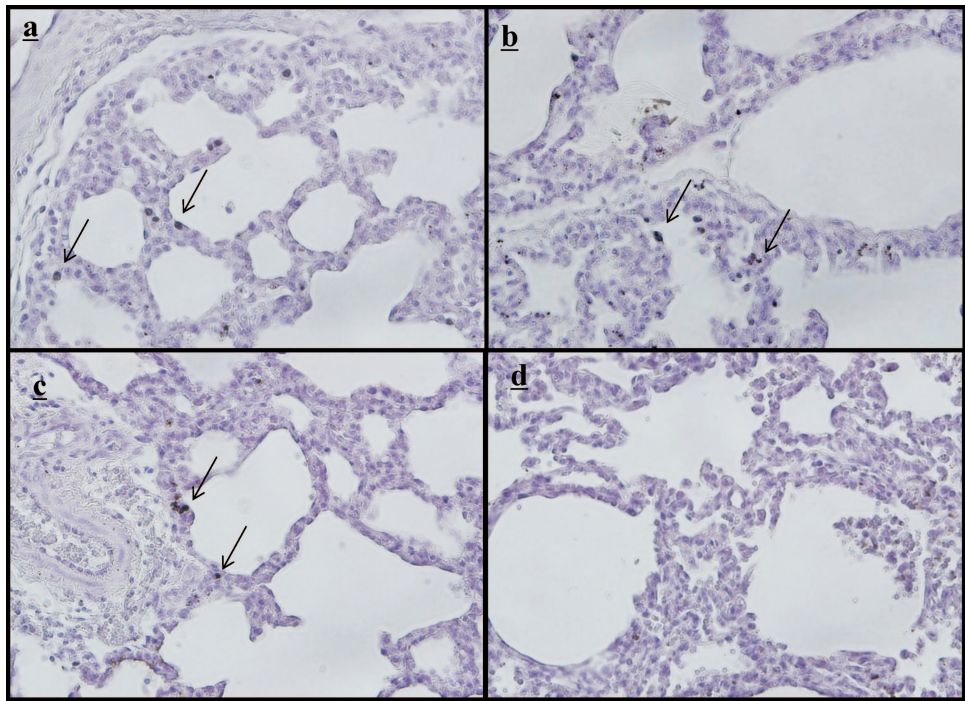


FIG. 3. Histologic lesions in pigs infected with porcine H5N2 viruses. Lung tissue sections were stained by immunohistochemistry using a polyclonal antibody against the nucleoprotein gene (anti-NP) of influenza virus to show influenza virus infection. (a) Sw/Kor/C12/08; (b) Sw/Kor/C13/08; (c) positive contact (Sw/Kor/CC13/08); (d) negative contact. Magnification, $\times 400$. The arrows indicate influenza virus-infected cells.

influenza isolates in this study had any deletion in the stalk region of the NA protein, both the catalytic and framework sites appeared to be conserved, and none contained mutations at sites known to confer resistance to neuraminidase inhibitors (15, 30, 52). However, a potential glycosylation site at position 402 (Asn) of the N2 protein was lost (Lys) in Sw/Kor/C12/08. Compared to the sequence of Ga/San Jiang/160/06, the two

isolates in this study had mutations at sites in the HA1 domain (R2Q, D155N, K170E, and N183D) and N2 proteins (Q5H and I241M). Furthermore, additional mutations could be noted for the surface proteins of Sw/Kor/C12/08 (in the HA1 domain, S107N and A185E; in the NA, N402K) and Sw/Kor/C13/08 viruses (in the HA1 domain, H103Y, D261Y, and L318P; in the NA, W403R), but none of these residues was

TABLE 4. Molecular analysis and comparison of amino acid sequences of different gene products of the swine H5N2 viruses with selected H5N1 isolates

Strain ^a	HA1 sequence at			NA stalk deletion	M2 sequence at aa:		NS sequence		PB2 sequence at aa 627
	aa 222 (226) ^b	aa 224 (228) ^b	Cleavage site (aa 323–330)		26	31	Deletion of aa 80–84	aa 92	
HK/483/97	Q	G	RERRRKKR	Yes	L	S	No	E	K
GS/437-4/99	Q	G	RERRRKKR	No	L	S	No	D	E
Ck/HK/YU822.2/01	Q	G	RERRRKKR	Yes	L	S	Yes	E	E
Dk/HK/821/02	Q	G	IERRRKKR	Yes	L	S	Yes	E	E
Dk/China/E319-2/03	Q	G	RE-RRRKR	Yes	L	S	Yes	E	E
Ck/Kor/ES03	Q	G	RE-RRRKR	Yes	L	S	Yes	E	E
Thailand/1/04	Q	G	RERRRKKR	Yes	—	—	—	—	E
Vietnam/1196/04	Q	G	RERRRKKR	— ^c	I	N	Yes	E	K
Ck/Kor/IS/06	Q	G	GERRRKKR	Yes	L	S	Yes	E	K
Qa/Kor/KJ4/06	Q	G	GERRRKKR	Yes	L	S	Yes	E	K
Env/Kor/W150/06	Q	G	GERRRKKR	Yes	L	S	No	E	K
Sw/Kor/C12/08	Q	G	RE----TR	No	L	S	No	D	E
Sw/Kor/C13/08	Q	G	RE----TR	No	L	S	No	D	E

^a The isolates in boldface are the Korean swine H5 viruses examined in this study.

^b The number in parentheses is the H3 numbering.

^c —, Sequence data not available.

TABLE 5. Amino acid sequence comparison of the H5N2 viruses isolated after experimental inoculation into pigs and ferrets^a

Isolate and gene	Position (aa)	aa found in:			
		Farm isolate	Pig	Contact pig	Ferret
Sw/Kor/C12/08					
PB2	144	R	R		K
	634	S	S		F
	741	S	S		F
NP	486	S	S		Y
NA	314	S	S		F
M	20	V	V		L
Sw/Kor/C13/08					
PB2	648	L	L	L	P
	653	S	S	S	F
	695	L	L	L	F
NA	314	S	S	S	F
NS1	112	T	T	T	A

^a The RNA of viruses recovered from inoculated pigs (including the contact pig infected with Sw/Kor/C13/08) and ferrets was extracted, reverse transcribed, and sequenced. Full sequences were compared against those of respective farm isolates using MegAlign of DNASTar package 5.0.

implicated previously for antigenicity or receptor-binding affinity.

Neither virus had an E-to-K mutation at position 627 of the PB2 protein, which was responsible for the high virulence of A/Hong Kong/483/97 in a mammalian host model (16). The avian-like PB2/627E present in the swine-like PB2 is common for all Korean swine influenza isolates and does not represent a K-to-E mutation in the PB2 genes of the isolates in this study. Neither of their M2 proteins had the necessary mutations at residue 31 (Ser to Asn) to confer amantadine resistance (17, 20), nor did they have glutamic acid at position 92 of the NS1 molecule for escaping host antiviral cytokine responses (46). To determine whether the pig-passaged H5 isolates had undergone further substitutions or mammalian-like adaptations, viruses recovered from experimentally inoculated pigs (including from the contact swine of Sw/Kor/C13/08) and ferrets were sequenced. Analysis showed that the sequences of viruses obtained from pigs bore the same sequences as the swine farm isolates. Interestingly, viruses recovered from ferrets incurred additional sequence differences indicative of continuous genetic evolution (Table 5). In summary, the molecular analysis of the viral proteins of the swine H5N2 viruses clearly showed that they maintained a low-pathogenicity form, and no mutations related to virulence, drug resistance, or a shift in host preference were acquired.

DISCUSSION

During our routine porcine virus surveillance in January and February 2008, two LPAIs of the H5N2 subtype (designated Sw/Kor/C12/08 and Sw/Kor/C13/08) were isolated for the first time from pigs in Korea. Viruses of the same subtype have been found among avian species in several countries, including the United States (31), Mexico (12), Italy (8), Nigeria (11), China (9), and Japan (35). However, there has been no report yet of infections of this virus subtype among swine. Initial homology comparisons of individual genes to viruses available

in the GenBank database showed that the two porcine viruses originally were derived from avian isolates and are almost identical to each other, except in four internal segments.

The phylogenetic analysis of the surface genes of the two porcine H5N2 viruses showed that both the HA and NA segments originated from a 2006 Chinese avian Ga/San Jiang/160/06 (H5N2)-like virus as a precursor (Fig. 2a and b). On the other hand, the internal genes might have originated from several different avian viruses purely belonging to the Eurasian lineage. These gene cascades of the avian H5N2 viruses that infected swine further reflect the dynamic influenza virus gene pool in this region, and new viruses are created by reassortment events that are very likely to occur in the field, exemplified by H5 viruses from southeastern China (9). However, phylogenetic analyses of the PB2, PA, NP, and M genes of the Sw/Ko/C13/08 virus revealed that they were descendants of a Korean H3N1-like virus (at least 99.5% similarities). In 2006, Shin et al. reported the presence in the Korean swine population of influenza viruses of the H3N1 subtype that were reassortants between human-like influenza viruses and recently circulating swine viruses (47). It is probable that the two porcine H5N2 viruses in this study originated from a common avian ancestor, but a coinfection with a swine H3N1-like virus in the same pig as a result of dual susceptibility facilitated an avian-like (HA, NA, PB1, and NS) or swine-like (PB2, PA, NP, and M) reassortant H5N2 virus.

Duan et al. reported that low-pathogenicity H5 subtype influenza viruses were isolated predominantly from migratory or sentinel ducks during the winter, were barely detected in market waterfowl, and were not found in terrestrial poultry in southern China from 2002 to 2005 (9). In our unpublished data, avian H5N2 viruses also were found in migratory birds in Korea but were absent in domestic poultry. Hence, the avian H5N2 viruses that infected Korean pigs may have come directly from migratory birds and not from domestic poultry products, as was suggested by the phylogenetic study, though the manner of such transmission remains to be resolved.

Although it is difficult to estimate when the avian-like H5N2 virus was first introduced into swine, some evidence suggests that the virus has been circulating for just a couple of years. First, the two porcine H5N2 isolates shared high amino acid and nucleotide sequence identities (>98.2%) in the HA and NA sequences with the Ga/San Jiang/160/06-like virus. The phylogenetic alignment of the surface genes further showed the close relationship between the isolates. Second, we found serologic evidence of avian H5N2 influenza virus infection from 2006 to the first quarter of 2008, but only in a small proportion of pigs (0.85%). The low seroprevalence and low swine-to-swine transmission of the purely avian-like H5N2 virus suggest that it did not spread significantly throughout South Korea. It also is possible that following transmission, successive infections of pigs were subclinical. Subsequently to successful cross-species transmission, spreading within the new host population usually requires a period of adaptation of the virus to that new host (55). Third, the PB2, PA, NP, and M genes of the Sw/Kor/C13/08 isolate had very high sequence similarities (98 to 99%) with Sw/Kor/CN22/06, as was shown phylogenetically, very likely indicating a recent reassortment event.

Some LPAI H5 viruses, aside from H7 viruses, could un-

dergo mutational changes to become highly pathogenic in avian species by several mechanisms and have been well documented (12, 21, 28, 40, 51). However, molecular analysis showed that none of the isolates in this study had any indications that they had acquired genetic mutations that would allow a shift in sialic acid receptor-binding preference, elevated pathogenicity, or increased drug resistance. We also could not predict whether such a virulence shift would occur if the viruses were allowed to circulate for an extended period of time in swine populations. Although both porcine viruses caused modest virus titers in the nasal swabs and showed moderate clinical signs, animal experiments showed that Sw/Kor/C13/08 was well adapted in swine and was more easily transmitted than Sw/Kor/C12/08. Between the Korean swine H5N2 viruses, the only apparent selective advantage of the Sw/Kor/C13/08 virus over the Sw/Kor/C12 virus is the acquisition of the swine-like segments. Therefore, it is interesting to speculate that the possession of the Sw/Kor/C13/08 isolate of the swine gene complements (i.e., the PB2, PA, NP, and M genes) conferred a selective advantage for transmissibility in swine hosts, as was shown in this study. Although our animal experiments showed that the purely avian-like H5N2 isolate was not transmitted to contact animals, it is not clear whether transmission would occur under field conditions (with the presence of bacterial coinfections and environmental stresses).

The surface genes (HA and NA) are the constant target of neutralizing antibodies, such that they largely define the genetic evolutions of influenza viruses. In contrast, there are no known selective pressures that could have driven the acquisition of specific swine-like internal genes. We could only hypothesize that the accumulation of acquired gene mutations on the avian progenitor of Sw/Kor/C13/08 defined its persistence in pigs, and that coinfection with the swine progenitor in the same host generated a reassortant progeny virus with a certain gene constellation effect facilitating transmission. There is increasing evidence that the viral polymerases play a major role in host adaptation (10). More recently, Manzoor et al. have demonstrated that a swine-derived PB2 gene conferred enhanced replicative potential to the reassortant virus that possessed seven genes of avian origin (33). It also should be noted that several researchers have proposed the NP gene as a determinant of host range that can either restrict or attenuate virus replication (44, 48, 54), thereby controlling the successful transmission of virus to a new host (2). Furthermore, the influenza virus HA gene was shown to have a preferential association with the M gene, and as such the evolution of the M gene may reflect host-specific adaptation (45, 54, 56). Overall, it will be interesting to investigate the individual contribution of these swine H3N1-derived internal viral genes with respect to transmissibility.

The isolation of two distinguishable H5N2 viruses in Korean swine in 2008 represents another significant event in the ability of pigs to act as intermediate hosts for low-pathogenicity H5 viruses. Just as in the cases of swine infections with avian H9N2 and H5N1 from nearby China, the isolated low-pathogenicity H5N2 viruses in this study may not be disregarded for their potential to cause human infections in the future. The shift from a purely avian-like swine virus to an avian-swine reassortant after apparent coinfection in pigs provides further evidence in the accumulating role of pigs as mixing vessels for

influenza viruses, enhancing the genetic evolution that bridges the gap between animal and human influenza viruses. It should also be noted that after a single passage to a mammalian host (ferret), some genes (most specifically the PB2 gene) already had acquired amino acid base changes. Such features of the avian-swine H5N2 porcine virus could be considered a potential model for pandemic highly pathogenic avian influenza (e.g., H5N1 and H7N7) virus outbreaks, in which viruses that were previously nontransmissible in a new host (e.g., humans) could also gain selective advantage by genetic reassortment with other strains of different lineages due to coinfection and through accumulated gene mutations. Although there are no known clinical implications of the avian-swine reassortant virus for pathogenicity to pigs or other species, including humans, at present, the efficient transmissibility of the relatively swine-adapted virus could facilitate virus spread, and association with disease outbreaks among swine populations could also be expected. Thus, it raises concerns for continued surveillance of yet another atypical influenza virus in pigs that may have the potential to cross host-species barriers.

ACKNOWLEDGMENTS

This work was supported by grant no. RO1-2005-000-10585-0 from the Basic Research Program of the Korea Science & Engineering Foundation and in part by a TBP grant from KRIBB (KGM3110912), by contract HHSN266200700005C with the National Institute of Allergy and Infectious Diseases, by Cancer Center support grant CA21765 from the National Cancer Institute, and by the American Lebanese Syrian Associated Charities (ALSAC).

We thank Taek-Kyu Oh and Eun Ho Lee for technical assistance.

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